Exhibit D

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VASCULAR NETWORKS WITHIN THE STROMA OF HUMAN LONG-TERM BONE MARROW CULTURES

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SUMMARY

The formation of branching and anastomosing vascular structures has been demonstrated within the stromal layers of human long-term bone marrow cultures (LTBMCs). Such organized vascular structures have not been described previously and may be of functional importance, as adherent granulocyte-predominant haemopoietic foci were more numerous in their interstices and margins than elsewhere in the stromal layers. Mature granulocytes were seen within vascular lumina in a few instances, possibly indicating an attempt to recapitulate the normal process of egress of neutrophils from bone marrow into the circulation. Immunostaining showed that the vascular structures expressed CD31, CD34, and CD105 (endoglin), which were not expressed by stromal fibroblastic cells. Collagen IV and laminin, expressed throughout the stroma, were present in increased amounts where vascular arrays were seen. In contrast, von Willebrand factor (VWF) and vascular cell adhesion molecule-1 (VCAM-1) were expressed equally by the vascular arrays and by stromal fibroblastic cells. Neither the arrays nor the general stroma expressed intercellular adhesion molecule-1 (ICAM-1), ICAM-2, ICAM-3, or endothelial leukocyte adhesion molecule-1 (ELAM-1).

KEY WORDS—human long-term bone marrow culture; immunohistochemistry; stroma; endothelium; CD31; CD34; CD105; granulopoiesis

INTRODUCTION

Long-term culture of bone marrow cells provides a variety of experimental models for the study of aspects of haemopoiesis. Using such models, it has been possible to investigate the roles of stromal cells, extracellular matrix, and growth factors in the proliferation and differentiation of haemopoietic progenitor cells.[1–3] Long-term bone marrow cultures (LTBMCs), however, are essentially two-dimensional and it is unclear to what extent they are able to reflect the complex spatial organization of haemopoiesis which is found *in vivo*.

In human bone marrow, granulopoiesis is organized radially around the margins of trabeculae and larger blood vessels.[4,5] Erythropoiesis and megakaryocyte maturation occur more centrally within intertrabecular spaces. Monopoiesis appears to occur in a dispersed fashion throughout such spaces,[6] despite origin from a precursor cell shared with the granulocyte series. Little is known about the spatial organization of stages of lymphopoiesis presumed to take place within human bone marrow.

The formation of discrete haemopoietic foci within confluent stromal layers in LTBMCs is believed to reflect the existence of microenvironmental niches in which growth factors, bound to extracellular matrix components, are made available to multipotential stem cells.[7,8] Specific adhesive interactions with stromal cells and/or extracellular matrix are also required for the proliferation and differentiation of haemopoietic cells. For instance, developing granulocyte precursors adhere to a bone marrow matrix protein called

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Table I—Antibodies used for immunostaining in this study, with sources and antigenic specificities

Antigen	Antibody	Source
α-Smooth muscle actin	Anti-αSMA	Sigma*
CD31	JC70a	K. Gatter, Radcliffe Infirmary, Oxford
CD34	QBEnd10	Novocastra Laboratories*
CD44	BRIC 128	D. Anstee, International Blood Group Reference Laboratory, Bristol
Collagen type IV	Anti-collagen IV	Dako*
ELAM-1 (CD62e)	CBL180	Cymbus Bioscience*
Endoglin (CD105)	CBL418	Cymbus Bioscience*
ICAM-1 (CD54)	CBL450	Cymbus Bioscience*
ICAM-2 (CD102)	Anti-ICAM-2	Bender Medsystems*
ICAM-3	KS/28	K. Gatter, Radcliffe Infirmary, Oxford
Laminin	Anti-laminin	Sigma*
Low-affinity nerve growth factor receptor (NGFR)	ME20.4	Amersham*
MHC class II	WR18	Wessex Regional Immunology Service, Southampton
VCAM-1 (CD106)	14C3	D. Haskard, ICRF, St. Bartholomew's Hospital, London
Von Willebrand factor	Anti-Factor VIII-RAg	Dako*

*Commercial source.

foci were also counted and assessed in an equal area of stroma distant from any vascular array in each LTBMC studied. The two-tailed Student's t-test was then used to assess the statistical significance of differences between the proportion of foci showing either predominantly granulocytic or predominantly monocytic differentiation in the vicinity of, and at a distance from, vascular structures.

RESULTS

Immunostaining for CD31, CD34, and CD105 (endoglin) revealed the presence of arborizing linear structures within the adherent cell layers of human LTBMCs (Fig. 1). In 2-week cultures, single elongated or stellate cells expressing these antigens were present (Fig. 2), which we interpreted as precursors of the structures seen in longer established cultures. From 4 weeks onwards, progressively more numerous and more complex branching arrays of such cells were found.

The branching structures were also highlighted in cultures immunostained for collagen IV and laminin, although these antigens were present elsewhere in the stroma at a lower-level of expression (Fig. 3). Cells within the arrays also expressed VWF and VCAM-1 moderately strongly, and MHC class II and CD44 weakly. They were not well delineated by demonstration of these antigens, however, as spindle-shaped and polygonal fibroblastic cells throughout the stroma, categorized as such by their morphology and immunostaining for several collagen subtypes, showed expression at similar levels of intensity. The general stroma and the arrays lacked expression of ICAM-1, ICAM-2, ICAM-3, and ELAM-1. A sub-population of large, polygonal fibroblastic cells in the stroma expressed α-smooth muscle actin (ASMA) with a pronounced 'stress-fibre' pattern of immunostaining, and another population, with a highly dendritic cell morphology, expressed low-affinity nerve growth factor receptor (NGFR).[11] Neither of these cell types, however, showed any preferential association with the branching arrays and the cells actually forming the arrays lacked expression of both ASMA and NGFR.

We interpreted these branching structures as representing attempts at blood vessel formation within LTBMCs. Observation of CD34-immunostained human LTBMCs by transmission laser microscopy with stereoscopic three-dimensional reconstruction of the image showed that branches of the arrays had a cord-like structure (see Fig. 1D). That they could become canalized was indicated by the occasional finding

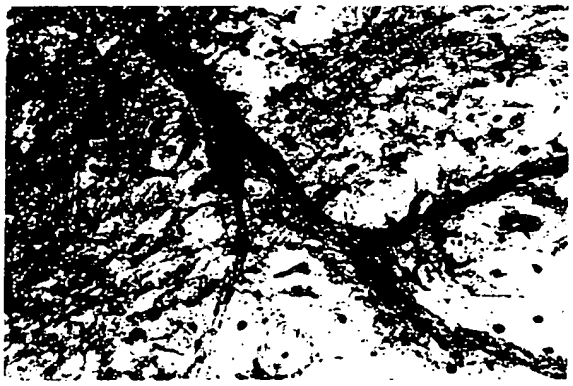

Fig. 3—Increased expression of collagen IV by vascular structures relative to expression within the general background stroma

Fig. 4—Mature neutrophil polymorphs appearing to occupy an intraluminal position within a CD34-positive vascular branch

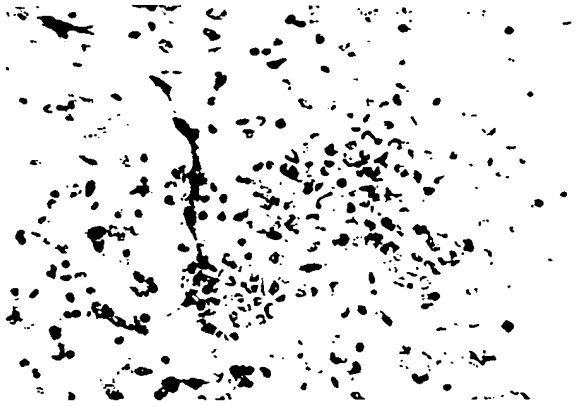

Fig. 5—Haemopoietic focus in the immediate vicinity of a CD34-positive vascular branch. High-power view to demonstrate differentiating granulocytes within the cluster of cells. Metamyelocytes of various sizes and degrees of nuclear folding, representing different degrees of maturation, are seen in addition to mature neutrophil polymorphs with segmented nuclei

the stroma. Monocyte-predominant foci showed no preferential association with these arrays. This was confirmed by quantitative analysis of 30 arrays from ten LTBMCs (Table II). Significantly more granulocyte-rich than monocyte-rich haemopoietic foci were found in the vicinity of arrays.

DISCUSSION

There has been no previous description of organized vascular structures within the stromal layers of human LTBMCs. Although some antigens known to be expressed by endothelial cells (VWF, VCAM-1 and CD44) were expressed by most stromal fibroblastic cells in addition to cells forming arrays in our cultures, these molecules were not present in geater concentration within the arrays and thus did not aid in their visualization. CD31, CD34 and CD105, however, were preferentially expressed by cells within arrays and revealed their distinctive morphology. Their pattern of arborizing and anastomosing multicellular cords is reminiscent of that seen in endothelial cultures grown in the presence of a supportive basement membrane gel.[13] Enhanced expression of collagen IV and laminin by the arrays is in keeping with the production of basement membrane by endothelial cells within them. Observation of intraluminal neutrophils in occasional cultures provides further evidence of the vascular nature of the arrays and indicates that they can become canalized.

The presence of branching vascular networks within the stroma of our human LTBMCs may be functionally relevant to the organization of haemopoiesis and, in particular, to the localization of granulopoiesis. Although we have found no evidence of adhesive ligands expressed reciprocally by granulocytes and these vascular structures,[11] haemopoietic foci with predominantly granulocytic maturation were seen in association with the arrays with a higher frequency than elsewhere in the stroma. This was not an exclusive association, as some granulocyte-predominant foci were found at sites distant from vascular arrays. Monocyte-predominant foci did not show a spatial association with these structures.

An alternative explanation for the association of clusters of granulocytic cells with vascular structures in human LTBMCs might be that they are not true haemopoietic foci, but that they represent

smooth muscle in their walls, although it has been postulated[16,17] that ASMA expression by stromal fibroblastic cells in human LTBMCs represents vascular smooth muscle differentiation. The vascular arrays also fail to express NGFR, an antigen present in perivascular cells around arterioles and venules within intact human bone marrow.[18] It is possible that the presence of distinctive polygonal ASMA-positive cells and dendritic NGFR-positive cells throughout the general stroma of human LTBMCs represents growth of vascular smooth muscle and pericytic cells which, under the culture conditions employed, are unable to organize with endothelia cells into three-dimensional structures. However, we have no evidence at present to confirm this suggestion and we found co-expression of some endothelium-associated antigens by stromal fibroblastic cells, raising questions as to their true nature.

The morphology of the vascular structures grown in our LTBMCs is suggestive of an array of capillaries. We hypothesize that the association of granulocyte-rich haemopoietic foci with these arrays might model some aspect of neutrophil egress from the bone marrow, rather than reflecting constraints regulating earlier stages of granulopoiesis. Functional studies of these interesting structures are now needed to clarify their relationship to vessel types found in bone marrow *in vivo* and to characterize their possible roles in haemopoiesis.

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REFERENCES

1. Quesenberry PJ, McNiece IK, Robinson BE, *et al.* Stromal cell regulation of lymphoid and myeloid differentiation. *Blood Cells* 1987; **13**: 137–146.
2. Campbell AD, Wicha MS. Extracellular matrix and the hematopoietic microenvironment. *J Clin Lab Med* 1988; **112**: 140–146.
3. Dexter TM. Haemopoietic growth factors. *Br Med Bull* 1989; **45**: 337–349.
4. Brown D, Gatter K. The bone marrow trephine biopsy: a review of normal histology. *Histopathology* 1993; **22**: 411–422.
5. Wilkins BS. Histology of normal haemopoiesis: bone marrow histology I. *J Clin Pathol* 1992; **45**: 645–649.
6. Wilkins BS, Jones DB. Cell–stroma interactions in monocytopoiesis. *FEMS Microbiol Immunol* 1992; **105**: 347–354.
7. Zipori D. Stromal cells form the bone marrow: evidence for restrictive role in regulation of haemopoiesis. *Eur J Immunol* 1989; **42**: 225–232.
8. Gordon MY, Riley GP, Watt SM, Greaves MF. Compartmentalisation of a haemopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. *Nature* 1987; **326**: 403–405.
9. Campbell AD. The role of haemonectin in the cell adhesion mechanisms of bone marrow. *Hematol Pathol* 1992; **6**: 51–60.
10. Patel VP, Lodish HF. The fibronectin receptor on mammalian erythroid precursor cells: characterisation and developmental regulation. *J Cell Biol* 1986; **192**: 449–456.
11. Wilkins BS, Jones DB. Immunohistochemical characterisation of intact adherent cell layers from human long-term bone marrow cultures. *J Pathol* 1994; **173**: 202A.
12. Hsu S-M, Raine L, Fanger H. Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *J Histochem Cytochem* 1981; **29**: 577–580.
13. Hughes SE, Del Buono R, Hall PA. Endothelial cell-extracellular matrix (ECM) interactions using ECV304. Role of acidic and basic FGFs in an *in vitro* model of angiogenesis. *J Pathol* 1993; **170**: 345A.
14. Coutinho LH, Gilleece MH, De Wynter EA, Will A, Testa NG. Clonal and long-term cultures using human bone marrow. In: Testa NG and Molineux G, eds. Haemopoiesis: A Practical Approach. Oxford: IRL, Oxford University Press, 1993; 75–106.
15. Allen TD, Dexter TM. The Living Haemopoietic Environment (Video). Manchester: Vector Television, 1991. (Copies available from Dr T. D. Allen, CRC Department of Structural Cell Biology, Paterson Institute, Christie Hospital NHS Trust, Manchester, M20 9BX, U.K.)
16. Charbord P, Lerat H, Newton I, *et al.* The cytoskelton of stromal cells from human bone marrow cultures resembles that of cultured smooth muscle cells. *Exp Hematol* 1990; **18**: 276–282.
17. Galmiche MC, Koteliansky VE, Briere J, Herve P, Charbord P. Stromal cells from human long term marrow cultures are mesenchymal cells that differentiate following a vascular smooth muscle differentiation pathway. *Blood* 1993; **82**: 66–76.
18. Cattoretti G, Schiro R, Orazi A, Soligo D, Colombo MP. Bone marrow stroma in humans: anti-nerve growth factor receptor antibodies selectively stain reticular cells *in vivo* and *in vitro*. *Blood* 1993; **81**: 1726–1738.